Journal of Sea Research xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Journal of Sea Research

journal homepage: www.elsevier.com/locate/seares

Short Communication

# A real-time PCR approach to detect predation on anchovy and sardine early life stages

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ARTICLE INFO

*Keywords:*
Early life stage mortality
Pelagic fish
Predation
Stomach contents
TaqMan probe
Mackerel larvae

ABSTRACT

Recruitment of sardine (*Sardina pilchardus* Walbaum, 1792) and anchovy (*Engraulis encrasicolus* Linnaeus, 1758) is thought to be regulated by predation of their eggs and larvae. Predators of sardine and anchovy can be identified by visual taxonomic identification of stomach contents, but this method is time consuming, tedious and may underestimate predation, especially in small predators such as fish larvae. Alternatively, genetic tools may offer a more cost-effective and accurate alternative. Here, we have developed a multiplex real-time polymerase chain reaction (RT-PCR) assay based on TaqMan probes to simultaneously detect sardine and anchovy remains in gut contents of potential predators. The assay combines previously described and newly generated species-specific primers and probes for anchovy and sardine detection respectively, and allows the detection of 0,001 ng of target DNA (which corresponds to about one hundredth of the total DNA present in a single egg). We applied the method to candidate anchovy and sardine egg predators in the Bay of Biscay, Atlantic Mackerel (*Scomber scombrus*) larvae. Egg predation observed was limited primarily to those stations where sardine and/or anchovy eggs were present. Our developed assay offers a suitable tool to understand the effects of predation on the survival of anchovy and sardine early life stages.

## 1. Introduction

Sardines (*Sardina pilchardus* Walbaum, 1792) and anchovies (*Engraulis encrasicolus* Linnaeus, 1758) are economically important pelagic species that are a focus of ecological research in support of stock assessment. As for other fish species, their early maturity and incorporation into the adult stock (first or second year – Ganias, 2014) make the annual recruitment a critical event in population dynamics. However, since mortality rates tend to decrease during development, it is generally assumed that survival during the early life (eggs and larvae) stages (ELS) controls recruitment rates (Aldanondo et al., 2016; Cowan and Shaw, 2002). Among the different variables described as factors determining mortality rates, predation has been identified as the most important (Lasker, 1981; McGurk, 1986; Albaina et al., 2010). Several studies have shown that fish eggs and larvae are a large component of the diet of many marine invertebrates (*e.g.* gelatinous zooplankton, copepods, chaetognaths, euphausids, amphipods, and snails) (*e.g.* Bailey and Houde, 1989), and that fish are also a major predator of other fish, particularly among the smaller size classes (Bax, 1998; Plirú et al., 2012).

In the Bay of Biscay, recent studies have successfully attempted to evaluate the predation pressure of adult fish and macrozooplankton on ELS of small pelagic species (Bachiller et al., 2015; Albaina et al., 2015). However, there is still an important lack of knowledge on the trophic relationships occurring among ELS of different species. For example, within this area, spawning periods for sardine and anchovy partially overlap spatially and temporally with that of Atlantic mackerel (*Scomber scombrus*) (Olaso et al., 2005). This, together with the increasing abundance of mackerel (Nøttestad et al., 2016) and its zooplanktivorous nature (Bachiller and Irigoien, 2013; Bachiller and Irigoien, 2015; Olaso et al., 2004, 2005), both, in the adult and larval stage (Conway et al., 1999), suggests that mackerel larvae could be exerting considerable predation pressure over sardine and anchovy ELS. Yet, to our knowledge comprehensive diet composition analyses of mackerel larvae are lacking. This is probably because visual examination of stomach contents (a common way to study diet and trophic interactions - King et al., 2008, although there are others - Symondson, 2002) is labour-intensive, time consuming, and may provide inaccurate data in the case of ELS, which are difficult to identify and are rapidly digested (Folkvord, 1993). Indeed, studies reporting presence of fish

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http://dx.doi.org/10.1016/j.seares.2017.06.009
Received 13 October 2016; Received in revised form 15 June 2017; Accepted 21 June 2017

eggs and larvae usually do not determine the species, and in some instances, not even the genus (Schooley et al., 2008).

DNA-based methods may represent an alternative for rapid and unambiguous identification of prey species in stomach contents. For example, metabarcoding (the taxonomic identification of a complex sample by sequencing and comparing a standardized DNA fragment (barcode) against a reference database) can provide the inventory of the species present in the stomach of a given predator. Consequently, to avoid amplification of the predator's DNA, this method requires utilization of blocking primers, which need to be designed specifically for each predator (Leray et al., 2013). For this reason, this method is recommended for the study of the prey composition of a reduced number of predator species. Alternatively, if the study of the range of predators that consume one or several prey species is required, methods such as digital Polymerase Chain Reaction (PCR) or quantitative or real-time PCR (qPCR or RT-PCR) are more cost effective. In particular, qPCR, which is able to cost-effectively detect residual DNA from visually imperceptible prey, has been proven successful as a means to study predation, and assays have been developed for the detection of a number of prey species (Taylor et al., 2002; Albaina et al., 2010) and tested in a variety of predators (Hunter et al., 2012; Fox et al., 2012).

Despite these methodological developments, until present, fisheries science has paid little attention to this technology in order to study ELS predation by fish larvae. Nonetheless, a more recent study has addressed predation on anchovy by macrozooplankton and two fish species using qPCR (Albaina et al., 2015). In order to expand this research and improve the understanding of the effects of potential predation on anchovy and sardine ELS, we have developed a real-time PCR based assay to detect DNA remains of sardine and anchovy simultaneously. The newly developed assay has been applied to test presence of anchovy and/or sardine in the stomachs of Atlantic mackerel larvae, a potential predator of these species' ELS.

## 2. Materials and methods

### 2.1. Sampling and DNA extraction

Tissue samples were taken from adult specimens of anchovy (*Engraulis encrasicolus)*, sardine (*Sardina pilchardus*), argentine (*Argentina sphyraena*), four-spot megrim (*Lepidorhombus boscii*), blackbellied angler (*Lophius budegassa*), angler (*Lophius piscatorius*), whiting (*Merlangus merlangus*), hake (*Merluccius merluccius*), mackerel (*Scomber scombrus*), horse mackerel (*Trachurus trachurus*) and pouting (*Trisopterus luscus*) collected from scientific surveys and commercial fisheries. Sardine and anchovy eggs were collected and sorted during the BIOMAN (ICES, 2016a) scientific data-gathering surveys, and Atlantic mackerel larvae during the TRIENAL (ICES, 2016b) survey in March, April and May at six stations in the Bay of Biscay, located between 43° 35′N-47° 45′N latitude and 2° 44′W-6° 15′W in longitude. Oblique hauls of a 330 μm mesh size Bongo plankton net attached to a 500 μm mesh size MIK (Methot Isaac Kidd) were used, and nets were deployed to 200 m or to 5 m above the bottom at shallower stations, at 2–2.5 nautical knots for 20 min. Sardine and anchovy eggs, as well as mackerel larvae, were sorted and stored individually in 96% ethanol immediately after collection. Larvae and tissue genomic DNA was extracted from ~20 mg of muscle tissue or from whole larvae using the Wizard® Genomic DNA Purification kit (Promega, WI, USA) and following manufacturer's instructions for “Isolating Genomic DNA from Tissue Culture Cells and Animal Tissue”. DNA from eggs was extracted following a protocol by Gleason and Burton (2012). Extracted DNA concentration was determined with the Quant-iT dsDNA HS assay kit using a Qubit® 2.0 Fluorometer (Life Technologies). DNA quality was assessed with the ND-1000 (NanoDrop) spectrophotometer and DNA integrity by electrophoresis on a 0.7% agarose gel.

**Table 1**
Primer and probe sequences for *Engraulis encrasicolus* (ANE), developed by Albaina et al. (2015) and *Sardina pilchardus* (PIL), generated in this study. Forward (F) and reverse (R) primer sequences, and probe (PROBE) sequences are shown together with the size of the expected PCR products.

| Primer/probe | Sequence 5′ to 3′ | Amplified region size |
|---|---|---|
| PIL_F | GACCTCCTGGGATTTGCAGT | 246 bp |
| PIL_R | GGTGTGAAGGATGGGGACGA | |
| PIL_PROBE | TTACCTCACTAGCCCTTT | |
| ANE_F | TTCTTACATGAATCGGAGGTATGC | 87 bp |
| ANE_R | GGAARATAGAGAAGTAGAGTAGCGATGCT | |
| ANE_PROBE | CGAACACCCATTCAT | |

### 2.2. Sardine and anchovy DNA detection assay development

The specific primers and probe targeting the cytochrome *b* (cyt b) gene were developed to detect sardine DNA. The cyt b gene was selected for being highly variable and allowing selection of small specific regions. All available sardine cyt b sequences ($n$ = 81) were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/), aligned with Clustal W (Thompson et al., 1997), and a consensus sequence was generated using Bioedit (Hall, 1999). Using the Primer Express 3.0 software (Applied Biosystems), forward and reverse primers were designed to amplify a 246 bp conserved region of the cyt b gene. None of the corresponding consensus sequence positions of forward and reverse primers or probe contained ambiguous bases. A TaqMan MGB (minor groove binding) probe was designed according to the requisites suggested by the manufacturer (Applied Biosystems). Both primers and probe were tested by BLAST (Basic Local Alignment Search Tool) similarity searches (http://ncbi.nlm.nih.gov/BLAST) against the EMBL and GenBank databases to verify the species-specificity of the sequences. The 5′ end of the sardine probe was then labelled with fluorescent dye VIC (Applied Biosystems), and the 3′ end with a non-fluorescent quencher. For the anchovy primer/probe set, we used a previously published design (Albaina et al., 2015) and labelled the probe with the fluorescent dye FAM (Applied Biosystems) while additionally labelling the 3′ end with a dark quencher.

### 2.3. Assay optimization

Each real-time PCR was performed as follows: 10 ng of DNA template, 5 μL of TaqMan FAST Advanced Master Mix (Applied Biosystems), 250, 500, 750 or 1000 nM of primers and 150, 200 or 300 nM of probe. $H_2O$ was added to adjust to 10 μL final volume. The real-time PCR was run under the following cycling conditions: one cycle at 25 °C for 30 s, one cycle at 95 °C 20 s, 40 cycles each of 95 °C for 3 s and 60 °C for 20 s and a final cycle of 25 °C for 20 s. Results were analyzed using the StepOne Software version 2.3 (Applied Biosystems) and threshold cycles ($C_t$, which stands for the cycle number at which the fluorescent signal passes a baseline threshold established by default by the software) were computed. Prior to combining the two probes into the same assay, primer and probe concentrations that provided the strongest and least unspecific signal were selected individually as the amplification of one target may influence the amplification of the other target (Herrero et al., 2011). Next, the real-time PCR with the two probes together was evaluated to obtain the best results in terms of specificity and sensitivity. For testing assay specificity, DNA from species inhabiting the Bay of Biscay was amplified separately using sardine or anchovy primers/probe sets under the above-mentioned conditions. Species tested were anchovy, sardine, argentine, four-spot megrim, blackbellied angler, angler, whiting, hake, mackerel, horse mackerel and pouting. One no template control (NTC) and two positive controls (one for sardine DNA and one for anchovy DNA) were used.